



TITLE:

Gene targeting technologies in rats: zinc finger nucleases, transcription activator-like effector nucleases, and clustered regularly interspaced short palindromic repeats.

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CITATION:

Mashimo, Tomoji. Gene targeting technologies in rats: zinc finger nucleases, transcription activator-like effector nucleases, and clustered regularly interspaced short palindromic repeats.. Development, Growth & Differentiation 2013, 56(1): 46-52

ISSUE DATE:

2013-12-27

URL:

<http://hdl.handle.net/2433/197304>

RIGHT:

This is the peer reviewed version of the following article: Mashimo, T. (2014), Gene targeting technologies in rats: Zinc finger nucleases, transcription activator-like effector nucleases, and clustered regularly interspaced short palindromic repeats. Development, Growth & Differentiation, 56: 46–52, which has been published in final form at <http://dx.doi.org/10.1111/dgd.12110>; この論文は出版社版ではありません。引用の際には出版社版をご確認ください。 ; This is not the published version. Please cite only the published version.

Gene targeting technologies in rats: ZFN, TALEN, and CRISPR

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Running title: Genome editing in rats

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Abstract

The laboratory rat has been widely used as an animal model in biomedical science for more than 150 years. Applying zinc-finger nucleases or transcription activator-like effector nucleases to rat embryos via microinjection is an efficient genome editing tool for generating targeted knockout rats. Recently, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated endonucleases have been used as an effective tool for precise and multiplex genome editing in mice and rats. In this review, the advantages and disadvantages of these site-specific nuclease technologies for genetic analysis and manipulation in rats are discussed.

Key words: clustered regularly interspaced short palindromic repeats, genome-editing, rats, transcription activator-like effector nucleases, zinc-finger nucleases

Introduction

Genetically modified animals that have been altered using gene targeting technologies are used as experimental models to perform functional analyses or various tests in biomedical research. In particular, knockout (KO) animals can help in understanding how a specific gene functions *in vivo*. The gene targeting technologies used to produce KO mice using embryonic stem (ES) cells were developed prior to 1990 (Mansour *et al.*, 1988, Capecchi, 1989a). Since then, KO mice have become major tools for functional gene analysis. Causative genes for specific human diseases have also been disrupted in mice to mimic human genetic disorders (Capecchi, 1989b, Smithies, 1993). In the post-genome era, the International Knockout Mouse Consortium (IKMC), which aims to comprehensively disrupt all protein-coding genes in the mouse genome using gene targeting technologies, is now progressing (Skarnes *et al.*, 2004, Nord *et al.*, 2006, Ayadi *et al.*, 2012). Furthermore, knock-in (KI) mice, in which genes are added or modified, or conditional knockout mice with spatial or temporal control of genetic inactivation, are widely used. Gene targeting technologies have become critical tools for understanding gene functions including the genetic basis of human diseases.

Until recently it was difficult to produce mammalian KO animals other than mice using gene targeting technologies, as germline-competent ES cells were available only for mice. However, this situation changed with the availability of newly developed gene targeting technologies, called engineered nucleases or “gene scissors”. These engineered nucleases, such as zinc finger nucleases (ZFNs) or transcription activator-like effector nucleases (TALENs), are very effective, as shown when treating embryos via microinjection to generate targeted KO mice and rats (Geurts *et al.*, 2009, Carbery *et al.*, 2010, Mashimo *et al.*, 2010, Meyer *et al.*, 2010, Urnov *et al.*, 2010, Cui *et al.*, 2011, Tesson

et al., 2011, Mashimo *et al.*, 2013, Sung *et al.*, 2013). In addition to rodents, sea urchins (Ochiai *et al.*, 2010), *Drosophila* (Beumer *et al.*, 2006, Beumer *et al.*, 2008), crickets (Watanabe *et al.*, 2012), killifish (Ansai *et al.*, 2012, Ansai *et al.*, 2013), and zebrafish (Doyon *et al.*, 2008, Meng *et al.*, 2008, Huang *et al.*, 2011, Sander *et al.*, 2011), as well as larger animals such as rabbits (Flisikowska *et al.*, 2011, Song *et al.*, 2013), and pigs (Watanabe *et al.*, 2010, Hauschild *et al.*, 2011, Carlson *et al.*, 2012), ~~and monkeys,~~ have been successfully modified using these enzymes. In this review, the advantages and disadvantages of these site-specific nuclease technologies are discussed in relation to genetic analysis and manipulation in animals, especially in rats.

Zinc-finger nucleases

ZFNs are chimeric proteins that consist of a specific DNA-binding domain that is made of tandem zinc finger-binding motifs fused to a non-specific cleavage domain of the restriction endonuclease *FokI* (Bibikova *et al.*, 2001, Porteus & Carroll, 2005, Wu *et al.*, 2007) (Fig. 1). As one zinc finger unit binds with 3-bp of DNA, 9–18 bp sequences can be specifically recognized by combining 3–6 different zinc finger units. By designing two zinc finger motifs on either side of 5–6 bp spacer sequences at a target region, the *FokI* nuclease combined with the zinc finger can introduce a double-strand break (DSB) within the 5–6 bp spacer sequences. Although the DSB is usually repaired via non-homologous end joining (NHEJ), an arbitrary deletion or deletion of base pairs often occurs during the repair process. Consequently, repair by NHEJ is mutagenic and mostly results in a loss-of-function mutation. Moreover, if DNA fragments homologous to the targeted sequences are co-injected with the nucleases, homologous recombination (HR) can occur, enabling insertion of a transgene or replacement of the homologous sequences at the targeted region,

which results in KI mutations. Therefore, artificially designed ZFNs can be used to generate KO or KI alleles at the targeted sequences via NHEJ or HR repair, respectively.

A summary of how to generate targeted KO rats using ZFNs is given in Fig. 1. Briefly, two ZFNs are designed across the spacer domain to recognize the targeted DNA sequences. Messenger RNAs are then transcribed *in vitro* from the two ZFN plasmids and injected into the male pronuclei of rat zygotes. Pronuclear stage embryos are collected from female rats that were superovulated by equine chorionic gonadotropin and human chorionic gonadotropin injection. The ZFN-injected embryos that differentiate into two cells are then transferred to the oviduct of pseudopregnant females. This method is based on a similar technique used to produce conventional transgenic animals (Palmiter *et al.*, 1982, Palmiter *et al.*, 1983, Mullins *et al.*, 1990), except that mRNA is used. The procedure for the micromanipulation of embryos is the same for all nucleases, including the below-mentioned TALEN and clustered regularly interspaced short palindromic repeats (CRISPR) enzymes.

The ZFN technology was first reported in the 1990s (Kim *et al.*, 1996, Chandrasegaran & Smith, 1999). From the 2000s, ZFNs have been developed for various mammalian cells (Bibikova *et al.*, 2001, Bibikova *et al.*, 2003, Porteus & Baltimore, 2003, Urnov *et al.*, 2005, Hockemeyer *et al.*, 2009), with nematode and zebrafish ZFNs developed in 2006 (Morton *et al.*, 2006) and 2008 (Doyon *et al.*, 2008, Meng *et al.*, 2008) respectively. The first genetic modification (KO) in rats was reported in 2009 (Geurts *et al.*, 2009). In model organisms where ES cells could not be used for gene modifications, this technology has been widely applied for generating genetically modified animals, especially in laboratory animals other than mice. Using this ZFN technology, we developed an interleukin-2 receptor gamma chain (*Il2rg*) KO rat (X-SCID) to investigate human X-linked severe combined immunodeficiency (X-SCID) (Mashimo *et al.*, 2010). SCID rats that are

deficient in the *Prkdc* gene and FSG (F344-*scid Il2rg*) rats that are simultaneously deficient in both the *Prkdc* and *Il2rg* genes have also been generated using the ZFN technology (Mashimo *et al.*, 2012). In contrast to the “leaky” phenotype of the SCID mouse, where immunoglobulins such as IgG, are detected in the blood, SCID rats did not show such a leaky phenotype (Mashimo *et al.*, 2012). These SCID rats can be used as hosts for xenotransplantation of human stem cells and tissues.

Transcription activator-like effector nucleases

ZFNs provide a straightforward strategy for targeted gene disruption in zygotes, resulting in rapid and cost-effective knockouts compared with conventional technology using ES cells. However, there are hurdles in terms of cost and protocols, making it difficult to establish ZFNs as a routine laboratory process. Recently, an artificial nuclease technology similar to ZFN, called TALENs, has been reported (Bogdanove & Voytas, 2011, Wood *et al.*, 2011, Mussolino & Cathomen, 2012, Joung & Sander, 2013). Natural TAL effectors are potent virulence proteins from plant-pathogenic *Xanthomonas* bacteria that are injected into eukaryotic host cells where they function as transcription factors (Bogdanove & Voytas, 2011). As fusions of TAL effectors to the *FokI* nuclease, TALENs can bind and cleave DNA in pairs (Fig. 1). Although the sequences recognized by ZF domains are limited in ZFNs, TAL effectors can recognize almost any sequence, except T at position 0. Simple and straightforward design and assembly strategies have been developed for rapid construction of TALENs (Carbery *et al.*, 2010, Cermak *et al.*, 2011, Sakuma *et al.*, 2013), providing a cost-effective targeted nuclease platform.

TALEN technology has also been reported in induced pluripotent stem cells (iPSCs) (Hockemeyer *et al.*, 2011), nematodes (Wood *et al.*, 2011), plants (Li *et al.*, 2012),

zebrafish (Huang *et al.*, 2011, Sander *et al.*, 2011), and rats (Tesson *et al.*, 2011). Although TALEN technologies seem to have advantages over ZFNs, there are also some ambiguous points that need to be clarified. For unknown reasons, the system appears to be less effective in rodent embryos. However, we recently showed that combined expression of exonuclease 1 (*ExoI*) with engineered site-specific TALENs provided highly efficient disruption of the endogenous gene in rat zygotes, and in the production of knockout rats for the albino (*Tyr*) gene (Mashimo *et al.*, 2013). The microinjection of TALENs with *ExoI* is an easy and efficient method of generating gene knockouts using zygotes, which increases the range of gene targeting technologies available to various species.

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) protein

The bacterial CRISPR/Cas system has recently been identified as an efficient gene-targeting technology in mammalian cells (Bassett *et al.*, 2013, Friedland *et al.*, 2013, Hwang *et al.*, 2013, Li *et al.*, 2013a, Li *et al.*, 2013b, Wang *et al.*, 2013). The system consists of a CRISPR that produces RNA components, along with the CRISPR-associated (Cas) nuclease protein. The CRISPR RNAs (crRNAs), containing short stretches of sequence homologous to specific target DNA, act as guides to direct Cas nucleases to introduce DSBs at the targeted DNA sequences. A synthetic chimeric guide RNA (gRNA) consisting of a fusion between crRNA and *trans*-activating crRNA (tracrRNA), directs Cas9 to cleave target DNAs that are complementary to the crRNA (Mali *et al.*, 2013). In addition to the ability to easily generate synthetic gRNAs, a significant advantage of the CRISPR/Cas system is that multiplex genes can be targeted simultaneously with multiple targeted

gRNAs. Furthermore, studies in mice have shown that homology-directed repair is preferentially activated over the NHEJ pathway when providing donor DNA templates (Wang *et al.*, 2013, Yang *et al.*, 2013). We have constructed the CRISPR/Cas architectures in rats, and applied it to embryos together with single-strand DNA oligonucleotides as donor templates, which efficiently generated targeted KI mutations in rats (unpublished data). Over the last decade, the emerging technology of next generation sequencing, and thereby genome wide association studies (Davey *et al.*, 2011, Biesecker & Spinner, 2013), has successfully identified numerous common SNPs in the human genome associated with important human diseases. As the functional testing of particular human SNP variants is a challenging proposition, accurate genome editing technologies are required for generating KI rats carrying equivalent mutations to human polymorphisms, rather than the KO models where entire coding genes are deleted. The CRISPR/Cas system provides sophisticated and flexible gene-targeting tools for generating suitable animal models of human diseases.

Advantages of the site-specific nuclease technologies

All of the artificial nuclease ZFN/TALEN/CRISPR technologies share the following advantages as compared with the conventional ES cell technology (Fig. 2). First and foremost, KO rats can be generated in a 4–6-month timeframe and with an efficiency of more than 20%. This is more favorable than the ES cell-based method for mice, which usually takes 12–18 months. Given the high rate of germ line transmission, preliminary phenotypic analysis can be performed on G1 animals after intercrossing the initial G0 founders, thereby saving time and effort. Second, gene targeting with artificial nucleases is not strain dependent (unpublished data), and accordingly can be performed with any inbred strain. This provides a straightforward strategy for directly employing targeted gene

disruption in existing strains, thereby bypassing tedious and time-consuming backcrossing steps that generally take 2–3 years to complete. Third, the artificial nuclease technologies can be used to induce a wide variety of allelic changes covering small or large deletions or insertions. It is also feasible to use targeted KI technologies that have thus far been inaccessible without rat ES cells. Since the technology does not rely on using species-specific ES cell lines, it may be possible to adapt it to other mammalian species such as pigs, cattle, and monkeys, where it is possible to harvest and manipulate fertilized embryos. The off-target effects are one of the biggest unknowns concerning the use of ZFN/TALEN/CRISPR technologies to modify the targeted genes (Radecke *et al.*, 2010, Fu *et al.*, 2013). It is always important to backcross the mutant lines with multiple generations to eliminate any off-target mutation and/or to validate the phenotypes with at least two independent lines.

The efficient production of inheritable genetically modified animals by artificial nuclease ZFN/TALEN/CRISPR technology will progress very rapidly. These genome-editing techniques will dramatically accelerate the development of advanced medical studies, drug design, and regenerative medicine, among other biomedical research applications, through the use of the huge number of genetically modified rats that are being produced.

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Figure legends

Figure 1. Gene targeting technologies with ZFNs, TALENs, and CRISPR/Cas in rats.

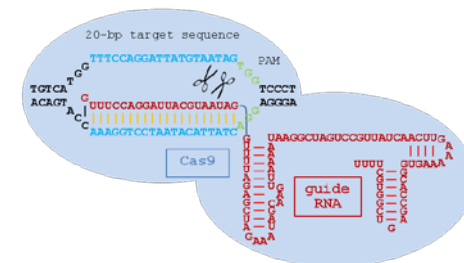
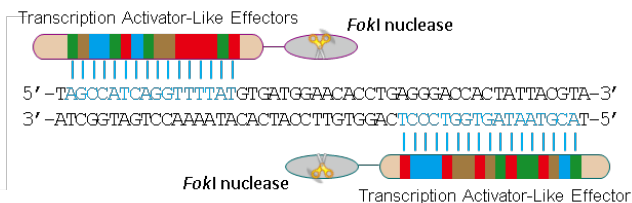
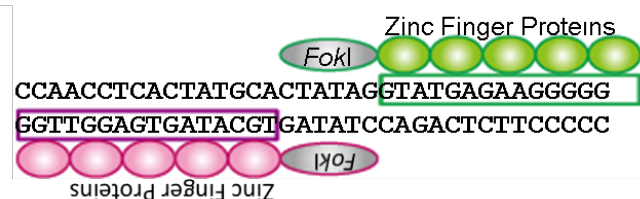
Schematic representation of the genetic engineering methods used for generating targeted knockout rats.

Figure 2. Various targeted genome modifications using ZFNs, TALENs, and CRISPR/Cas in animals.

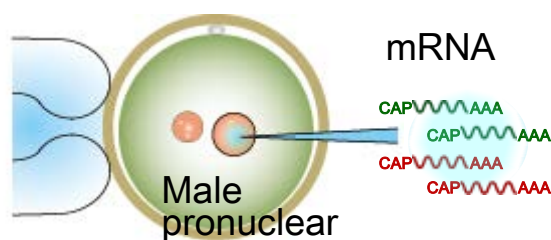
Zinc-finger nucleases (ZFNs)

TAL effector nucleases (TALENs)

CRISPR/Cas



Microinjection of ZFN/TALEN/CRISPR into rat embryos



Microinjection

Transfer



Pseudopregnant
foster mothers

Deriver

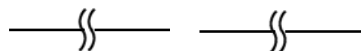


Pups (Founder)

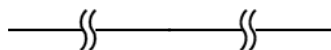
Figure 1

Multiplex knockout

- Different chromosomes

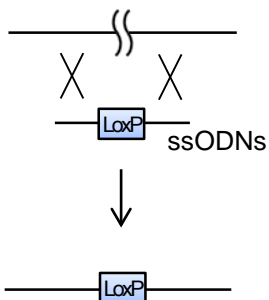


- Same chromosome

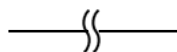


Conditional (flox)

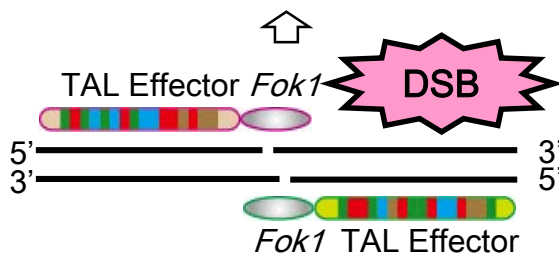
- Short insertion



Knockout



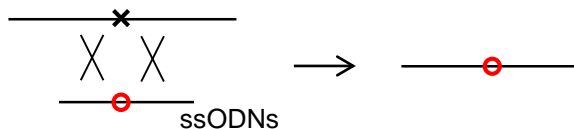
NHEJ repair



HR repair

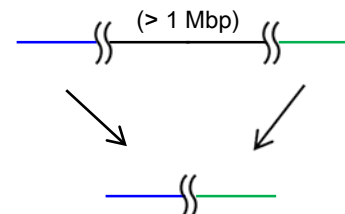
Knock-in

- SNP exchange



Large deletion

- Large deletion



Gene integration

- Cassette integration

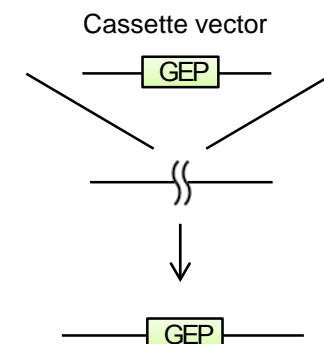


Figure 2